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Final Progress Report

Bioconjugation and Delivery of Magnetic Nanoparticle Probes

Research Supported by a Subcontract from MagneSensors

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Gang Bao, PhD
Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332

Personnel in Bao Lab: Dr. Leslie LaConte

Contacts within MagneSensors: Tom Pisanic, Dr. Kai Yang, Dr. Mark DiIorio

Summary

The Bao lab carried out fluorescence labeling and cellular delivery of magnetic nanoparticle probes, as indicated in the subcontract. Magnetic nanoparticles received from MagneSensors were labeled with a fluorescent dye and conjugated to a cell-permeating peptide for delivery into Hela cells in culture. Some of these particles were also conjugated to an antibody for tubulin, in an effort to immobilize the particles once delivered into the cell. Magnetic Nanoparticles were delivered into cells and imaged at the Bao lab at Georgia Tech. When incubated with cells at high concentrations, nanoparticles were observed to agglomerate on the surface of cells. When incubated with cells at low concentrations, nanoparticles were observed inside cells, both in large, micron-sized clusters, and as smaller, individual dots. Some nanoparticle batches were also returned to MagneSensors to evaluate the possibility of real-time quantification of intracellular binding using a superconducting quantum interference device (SQUID) magnetometer. Some of the nanoparticle batches were reported to have dissolved en route to MagneSensors, possibly due to the presence of PBS. This effect was not observed at Georgia Tech.

Nanoparticle Labeling and Conjugation

Fluorescence Labeling of Magnetic Nanoparticles

Streptavidin-Captivate™ ferrofluid conjugate (Molecular Probes, Invitrogen Corp., Carlsbad, CA), 0.5 mg Fe/mL, was concentrated either with a Microcon® YM10 centrifugal filter unit or by magnetic separation using a small neodymium magnet to improve labeling efficiency and change into labeling buffer. Labeling buffer was phosphate buffered saline (PBS) with 10% 1M sodium bicarbonate. The fluorescent label used was amine-reactive Alexa 546 (Molecular Probes). Alexa 546 was reacted at 8X molar excess the molar concentration of streptavidin on the surface of the particle (according to Molecular Probes, there are approximately 2500 streptavidin per particle). Reaction was done at room temperature with gentle shaking for two hours and then allowed to continue at 4°C overnight. Particles were washed with excess PBS to remove unreacted dye until supernatant had little residual fluorescence at 546 nm. Washes were performed in YM10 Microcons or with magnetic separation.

Conjugation of Tat Peptide and Anti-Tubulin Antibody

Biotinylated Tat Peptide (N terminus-TyrGlyArgLysLysArgArgGlnArgArgArg-Lys-Ahx-biotin) and biotinylated tubulin antibody were reacted with fluorescently labeled, streptavidin-coated Captivate. The actual conjugation procedure consisted of simply mixing the labeled Captivate gently (with vortexer) with the desired concentration of Tat, Ab or Tat and Ab mixture for one hour at room temperature. Several different concentrations were explored to optimize delivery and intracellular immobilization. For Tat peptide, concentrations ranged from 10 Tat peptides per calculated biotin site to 2 Tat peptides per magnetic particle. For the tubulin antibody, concentrations ranged from 3 antibodies per calculated biotin site to 2 antibodies per particle. Some reactions were performed in the presence of biotin (ranging from 1.5 X to 30X excess of the biotin sites) to avoid crosslinking when reacting with the multiply-biotinylated antibody.

Delivery of Labeled Captivate into Cells

Captivate labeled with Alexa 546, Tat peptide, and/or tubulin antibody were incubated with Hela cells to determine delivery efficiency and immobilization properties. Alexa-Captivate-Tat-(Ab) was mixed with Hela DMEM media in ratios ranging from 1:4 to . Medium was removed from the well of a 4-well plate and replaced with the Alexa-Captivate media and incubated for one hour at 37°C. After incubation, cells were washed with OptiMem (a reduced serum medium that lessens autofluorescence) and observed under a light microscope, a fluorescent microscope (40X and 100X) and sometimes a confocal fluorescent microscope.

Results and Discussion

The initial experiment for delivering Alexa-Captivate-Ab-Tat nanoparticles were carried out using nanoparticles that reacted with approximately 10 Tat peptides per biotin site; they were delivered into cells at high concentrations (1:4 or 1:8 Captivate to media ratio). Under both the light microscope and fluorescent microscope, it was apparent that these particles were clustered (micron-sized aggregates) all over the outside surface of the cell, but were not removable with washing. A control delivery experiment was performed by incubating with Hela cells the unlabeled Captivate without Tat conjugated to the surface, and no such aggregation was observed. However, when unlabeled Captivate was conjugated to Tat and then incubated as described above, micro-sized iron oxide clusters were visible on the surface of the cells in the light microscope.

To eliminate clustering on the surface of cells, two concurrent approaches were used. The amount of Tat peptide conjugated to the surface of the Captivate was dramatically reduced (roughly two Tat per particle) and the concentration of Alexa-Captivate-Tat was also reduced (1:30 Captivate:media). This preparation showed no evidence of cell surface aggregation, and imaging with the fluorescent microscope revealed evidence of Alexa-Captivate inside the Hela cells. Many large, bright aggregates (micron-sized) were visualized. Dimmer, smaller (submicron) fluorescent spots were also observed, suggesting the presence of two classes of internalized Captivate particles: (1) aggregates and (2) individual particles.

Labeled, conjugated particles were shipped to MagneSensors in California from Georgia Tech for SQUID examination. Upon receipt of the first package of labeled particles, the researchers from MagneSensors inspected the conjugated particles and reported that the particle concentration was significantly reduced from the original concentration shipped, i.e. the particles were likely dissolving over time. This was not observed in the Bao lab at Georgia Tech. After discussion with Molecular Probes, the MagneSensors researchers decided that PBS at low particle concentration might lead to nanoparticle dissolution.

A final batch of particles was prepared for MagneSensors, prepared in sodium bicarbonate buffer with two different concentrations of Tat peptide, based on experiments performed at MagneSensors. A High-Tat preparation contained approximately 1000 Tat peptides per particle, 10 antibodies per particle, and 3X excess biotin per binding site. A Low-Tat preparation differed only in that it contained 10 Tat peptides per particle. In an effort to rule out any role that the Alexa dye might be playing in the dissolution of particles, unlabeled Captivate was also conjugated for both a High-Tat and Low-Tat preparation. It was not clear whether or not these preparations yielded good results.